

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
31 December 2003 (31.12.2003)

PCT

(10) International Publication Number
WO 2004/001057 A1

(51) International Patent Classification⁷: C12P 23/00, (74) Agents: TEIPEL, Stephan et al.; Lederer & Keller, C12N 9/10 Prinzregentenstrasse 16, 80538 München (DE).

(21) International Application Number: PCT/EP2003/003742

(22) International Filing Date: 10 April 2003 (10.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 02013784.0 21 June 2002 (21.06.2002) EP

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/001057 A1

(54) Title: PROCESS FOR PRODUCING CAROTENOIDS

(57) Abstract: The present invention relates to a biological process for producing carotenoids utilizing a microorganism which is capable of producing carotenoids and belonging to the genus *Xanthophyllomyces* (*Phaffia*) in the presence of an inhibitor for biosynthesis of sterols from farnesyl pyrophosphate.

Process for producing carotenoids

The present invention relates to a biological process for producing carotenoids utilizing a microorganism which is capable of producing carotenoids and belonging to the genus *Xanthophyllomyces (Phaffia)* in the presence of an inhibitor for biosynthesis of sterols from 5 farnesyl pyrophosphate (hereinafter referred to as FPP).

Over 600 different carotenoids have been described from carotenogenic organisms found among bacteria, yeast, fungi and plants. Currently only two of them, beta-carotene and astaxanthin are commercially produced in microorganisms and used in the food and feed industry. These carotenoids are industrially important as natural pigments and as functional substances for human health with their powerful antioxidant properties. Moreover, 10 from a commercial prospect, there is an increasing demand for astaxanthin as a coloring reagent especially in the fish farming industry, such as salmon farming, because astaxanthin imparts a distinctive orange-red coloration to the fish and contributes to consumer appeal.

15 Other carotenoids, for example, lycopene, zeaxanthin, canthaxanthin, and beta-cryptoxanthin, are also industrially important as natural pigments and as antioxidants. Lycopene is shown in experiments performed *in vitro* to quench singlet oxygen efficiently. Lycopene inhibits lipid peroxidation, and serum levels of this carotenoid inversely related to the risk of cancer in the pancreas and cervix. The red color of fruits and vegetables such as tomatoes, pink grapefruit, the skin of red grapes, watermelon and red guavas is due to lycopene. 20 Other dietary sources include papaya and apricots. Zeaxanthin is a yellow-colored carotenoid, which is an oxidized hydroxy derivative of beta-carotene. Zeaxanthin is abundant in spinach and corn and many other plant species. This is a strong antioxidant and found also in the retina. It is widely believed that zeaxanthin acts to filter and shield harmful blue

light from the eye and protect against age-related macular degeneration. Canthaxanthin is a red-colored carotenoid, found in many plants and animals. It is used for pigmentation of egg yolk, broiler, and farmed trout, and used in foods and cosmetics requiring a more orange-red color. Canthaxanthin also functions as a singlet oxygen quencher and free radical deactivator. Beta-cryptoxanthin is a yellow-colored carotenoid found in oranges, mango, papaya, squash and many other fruits. Beta-cryptoxanthin is recognized also as a strong antioxidant useful for prevention from cancer.

Among the microorganisms which are capable of producing remarkable amounts of carotenoids, *Phaffia rhodozyma* is one of the most well-known carotenogenic strains. This yeast 10 strain produces astaxanthin and is one of the few microorganisms which are currently used to provide astaxanthin in the food and feed industry. In a recent taxonomic study, a sexual cycle of *Phaffia rhodozyma* was revealed and its telemorphic state was designated under the name of *Xanthophyllomyces dendrorhous* [Golubev, Yeast 11:101-110 (1995)]. In the present description, the widely recognized name, *Phaffia rhodozyma* is used.

15 Several studies to increase the level of carotenoids production by microorganisms including, for example, construction of recombinant microorganisms which were genetically engineered to obtain the ability to produce a remarkable amount of several carotenoids using appropriate host strains such as *Escherichia coli*, *Saccharomyces cerevisiae*, or *Candida utilis* [Misawa et al., J. Bacteriol. 172:6704 (1990); Yamano et al., Biosci. Biotech. Biochem. 20 58:1112 (1994); Miura et al., Biotechnol. Bioeng. 58:306 (1998)], strain improvement to obtain hyper-producers, for example, of astaxanthin from *Phaffia rhodozyma* [Johnson et al., Critical Reviews in Biotechnology 11:297-326 (1991)], and process improvement to optimize the fermentation process, for example, for astaxanthin production by *Phaffia rhodozyma* (US 5,972,642) have been conducted. It was described in US 5,356,809 that 25 addition of antimycin or another inhibitor of the main respiratory chain to *Phaffia rhodozyma* cells enhanced the astaxanthin production. But this is not a much efficient or convenient method. Clearly, there is still a need for a simple and efficient method of increasing the production yields of carotenoids.

One embodiment of the present invention is a biological process for producing 30 carotenoids which comprises cultivating a microorganism which is capable of producing carotenoids in the presence of an inhibitor for biosynthesis of sterols from FPP, in an aqueous nutrient medium under aerobic conditions.

It is more preferable to use highly carotenogenic microorganisms, e.g. those belonging to the genus *Xanthophyllomyces* (*Phaffia*). Thus, a preferable example of the said micro-

organism of the present invention is a microorganism belonging to the genus *Xanthophyllomyces* (*Phaffia*). A preferable strain of *Xanthophyllomyces* (*Phaffia*) of the present invention can be obtained from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, U.S.A. as *Xanthophyllomyces* (*Phaffia*) ATCC96594 (re-
5 deposited under the accession No. ATCC 74438 on April 8, 1998 pursuant to the Budapest Treaty).

The present invention is thus particularly concerned with a biological process for producing carotenoids which comprises cultivating a microorganism which is capable of producing carotenoids and belonging to the genus *Xanthophyllomyces* (*Phaffia*) in the presence of an inhibitor for biosynthesis of sterols from FPP, and a substrate for
10 producing carotenoids in an aqueous nutrient medium under aerobic conditions, and isolating the resulting carotenoids from the cells of said microorganism or from the cultured broth.

Especially, it will be more preferable, for the said biological process of the present invention,
15 to inhibit the first reaction of the sterol pathway, which is catalysed by squalene synthase.

Squalene synthase (also referred to in the art as squalene synthetase) inhibitors are reported to lower cellular sterol levels. Squalene synthase is an enzyme which catalyses the first committed step of sterol biosynthesis. Two molecules of FPP are condensed to form
20 squalene.

Thus, a further embodiment of the present invention is a biological process for producing carotenoids which comprises cultivating a microorganism which is capable of producing carotenoids in the presence of an inhibitor for squalene synthase.

Several kinds of squalene synthase inhibitors have been reported [Biller et al., Current
25 Pharmaceutical Design 2:1-40 (1996)]. There have been reported roughly three groups of squalene synthase inhibitors, ammonium ion based squalene synthase inhibitors, phosphorus containing FPP mimetics, and carboxylate-based inhibitors. Any kinds of squalene synthase inhibitors are preferable for the present invention. One of the preferable examples of such squalene synthase inhibitors is an ammonium ion based squalene synthase inhibitor.
30

Further, one of the preferable examples of the ammonium ion based squalene synthase inhibitor is a phenoxypropylamine-type squalene synthase inhibitor [Brown et al, Journal of Medicinal Chemistry 38:4157-4160 (1995)]. A large number of phenoxypropylamine-

type squalene synthase inhibitors including, for example, [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, N-isopropyl-3-(4-acetamido-2-allylphenoxy) propylamine, N-methyl-N-isopropyl-3-(4-acetamide-2-allylphenoxy) propylamine, N-cyclopentyl-3-(4-acetamido-2-allylphenoxy) propylamine, N-cyclobutyl-3-(4-acetamide-2-allylphenoxy) propylamine, N-isopropyl-3-(2-allyl-4-butyramidophenoxy) propylamine, N-isopropyl-3-(4-acetamido-2-propylphenoxy) propylamine, and N-isopropyl-3-(4-acetamido-2-allylphenoxy)-1-methylpropylamine, or biologically acceptable salt thereof, have been known (Brown et al., supra).

10 The preferable squalene synthase inhibitor used in the present Examples is [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, N-isopropyl-3-(4-acetamido-2-allylphenoxy) propylamine and N-methyl-N-isopropyl-3-(4-acetamide-2-allylphenoxy) propylamine.

Carotenoids are normally produced by cultivating a carotenogenic microorganism in a medium which comprises suitable macro- and micronutrients for the cells, such as molasses, saccharose or glucose as a carbohydrate source for cell growth and also as a substrate for producing carotenoids, and nitrogen sources such as corn steep liquor, yeast extract, diammonium sulphate, ammonium phosphate, ammonium hydroxide or urea, phosphorus sources such as ammonium phosphate and phosphoric acid and added micro-nutrients or mineral salts such as magnesium sulphate, zinc sulphate and biotin or desthiobiotin.

The preferable conditions for cultivation are a pH range from 4 to 8 and a temperature range from 15 to 26 °C for 24 to 500 hours. The more preferable conditions for cultivation are a pH range from 5 to 7 and a temperature range from 18 to 22 °C for 48 to 350 hours.

25 In the cultivation, aeration and agitation usually give favorable results for the production of carotenoids.

In the present invention, the inhibitor for sterol biosynthesis from FPP is added to the medium. Suitably, the concentration of the inhibitor is varied based on the species of inhibitor and microorganism used for the carotenoids production, e.g. in a range of concentration that gives less than 50 % reduction of the cell growth in the carotenoids producing conditions. A more preferable concentration of the inhibitor may be in the range of concentration that gives less than 30 % reduction of the cell growth.

In the present invention, the inhibitor for sterol biosynthesis from FPP can be added to the medium at any period of the cultivation.

Carotenoids produced by cultivating a carotenogenic microorganism using the methods of the present invention, can be isolated either from the medium, in the case they are secreted into the medium, or from the cells of the microorganism and, if necessary separated from other carotenoids that may be present in case one specific carotenoid is desired, by 5 methods known in the art [e.g., Carotenoids Vol IA: Isolation and Analysis, Britton et al., Birkhäuser Verlag, Basel (1995)].

Carotenoids produced in accordance with the present invention can be used in a process for the preparation of food or feeds. A man skilled in the art is familiar with such processes. Such compound foods or feeds can further comprise additives or components 10 generally used for such purpose and known in the state of the art.

The following Examples further illustrate the present invention, but these are not thereby limiting the scope of the invention.

The squalene synthase inhibitor used in the Examples is [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, one of the series of phenoxypropylamine-type inhibitors. This 15 compound was synthesized according to methods described by Brown et al. (supra): 3-Allyl-biphenyl-4-ol was prepared from biphenyl-4-ol (product code H7751, Sigma, USA) by reaction with allyl bromide and potassium carbonate in butan-2-one, and thermal rearrangement. 3-Allyl-biphenyl-4-ol was reacted with 1,3-dibromopropane and potassium carbonate in butan-2-one, and subsequent reaction with isopropylamine in 2- 20 propanol gave [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine.

Example 1: Effect of addition of the squalene synthase inhibitor on the cell growth of *Phaffia rhodozyma*

Phaffia rhodozyma ATCC96594 was inoculated into YPD medium (DIFCO, Detroit, U.S.A., 10 mL in tube) and cultivated by shaking at 20°C for 2 days. 0.5 mL of the culture 25 was inoculated into fresh YPD medium (10 mL in tube) containing 30 g/L of glucose and 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 µg/mL, respectively, of [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, and cultivated by shaking at 20°C for 5 days.

An aliquot of the culture was withdrawn occasionally during the cultivation, and optical density at 660 nm was measured by using UV-1200 photometer (Shimadzu Corp., Kyoto, 30 Japan) for analysis of the cell growth. The results are shown in Table 1.

Table 1

[3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine [μg/mL]	Cell growth (OD at 660 nm)			
	Day 1	Day 2	Day 3	Day 5
0	4.4	15.9	20.1	19.0
0.2	4.5	16.7	20.1	19.3
0.5	4.4	15.6	20.4	19.4
1.0	4.0	15.0	20.0	19.5
2.0	3.6	15.0	19.5	19.0
5.0	2.9	12.3	18.8	18.0
10.0	2.0	5.6	14.7	16.9
20.0	1.2	1.7	2.7	7.0

The cell growth was not affected when the concentration of the squalene synthase inhibitor was not higher than 2.0 μg/mL. About 23 % inhibition of the cell growth was observed at 5 Day 2 by 5.0 μg/mL of the inhibitor.

Example 2: Effect of addition of the squalene synthase inhibitor on the astaxanthin production by *Phaffia rhodozyma*

Phaffia rhodozyma ATCC96594 was inoculated into YPD medium as in Example 1 and cultivated by shaking at 20°C for 2 days. 2.5 ml of the culture were inoculated into fresh YPD medium (50 mL in flask) containing 22 g/L of glucose and 0, 0.5, 1.0, 2.0, and 5.0 μg/mL, respectively, of [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, and cultivated by shaking at 20°C for 7 days. On Day 2 of the cultivation, 50 g/L of glucose was added to the culture. Addition of the inhibitor in the middle of the cultivation was also tested. An aliquot of the culture was withdrawn at Day 4 and Day 7 of the cultivation, and optical density at 660 nm (by using the same method described in Example 1) and astaxanthin content in the culture were measured.

For analysis of the content of astaxanthin, the withdrawn broth was mixed with a solvent mixture (ethyl alcohol, hexane and ethyl acetate) and carotenoids were extracted from the cells of *Phaffia rhodozyma* by vigorous shaking with glass beads. After extraction, disrupted cells and glass beads were removed by centrifugation and the resultant supernatant

was analyzed by HPLC for the astaxanthin content. The HPLC conditions used were as follows:

HPLC column: Chrompack Lichrosorb si-60 (4.6 mm, 250 mm)
 Temperature: room temperature
 5 Eluent: acetone / hexane (18/82) add 1 ml/L of water to eluent
 Injection volume: 10 µl
 Flow rate: 2.0 ml/minute
 Detection: UV at 450 nm

A reference sample of astaxanthin was obtained from Hoffmann La-Roche (Basel, Switzerland). The results are shown in Table 2.

Table 2

[3-(3-allyl-biphenyl-4-yloxy)- propyl]-isopropyl-amine [µg/mL]	Astaxanthin content*		Cell growth (OD at 660 nm)	
	Day 4	Day 7	Day 4	Day 7
0	69	100	40.7	40.0
0.5	75	114	39.6	39.8
1.0	77	118	40.3	39.8
2.0	75	125	38.2	39.3
5.0	67	129	40.2	39.5
5.0 **	84	131	37.4	37.9

* Relative values calculated as the astaxanthin content of Day 7 without inhibitor to be 100.

** Cultivation was started without inhibitor, and 5.0 µg/mL of inhibitor was added at Day 15. 2 of the cultivation.

Astaxanthin production was enhanced in all conditions tested. The best result was obtained when the inhibitor was added at Day 2 of the cultivation, but addition of the inhibitor from the beginning of the cultivation was also effective on the astaxanthin production.

Claims

1. A biological process for producing carotenoids which comprises cultivating a micro-organism which is capable of producing carotenoids in the presence of an inhibitor for biosynthesis of sterols from farnesyl pyrophosphate, in an aqueous nutrient medium under aerobic conditions.
2. A biological process for producing carotenoids which comprises cultivating a micro-organism which is capable of producing carotenoids and belonging to the genus *Xanthophyllomyces* (*Phaffia*) in the presence of an inhibitor for biosynthesis of sterols from farnesyl pyrophosphate, a substrate for producing carotenoids in an aqueous nutrient medium under aerobic conditions, and isolating the resulting carotenoids from the cells of said microorganism or from the cultured broth.
3. The process according to claim 2, wherein the microorganism is *Xanthophyllomyces dendrophorus* (*Phaffia rhodozyma*) ATCC96594.
4. The process according to claim 1 or 2, wherein the inhibitor for biosynthesis of sterols from farnesyl pyrophosphate is selected from the group consisting of squalene synthase inhibitors.
5. The process according to claim 4, wherein the squalene synthase inhibitor is selected from the group consisting of ammonium ion based squalene synthase inhibitors.
6. The process according to claim 5, wherein the ammonium ion based squalene synthase inhibitor is selected from the group consisting of phenoxypropylamine-type squalene synthase inhibitors.
7. The process according to claim 6, wherein the phenoxypropylamine-type squalene synthase inhibitor is selected from [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, N-isopropyl-3-(4-acetamido-2-allylphenoxy) propylamine, N-methyl-N-isopropyl-3-(4-acetamide-2-allylphenoxy) propylamine, N-cyclopentyl-3-(4-acetamido-2-allylphenoxy) propylamine, N-cyclobutyl-3-(4-acetamide-2-allylphenoxy) propylamine, N-isopropyl-3-(2-allyl-4-butyramidophenoxy) propylamine, N-isopropyl-3-(4-acetamido-2-chlorophenoxy) propylamine, N-isopropyl-3-(4-acetamido-2-propylphenoxy) propylamine, and N-isopropyl-3-(4-acetamido-2-allylphenoxy)-1-methylpropylamine, and biologically acceptable salts thereof.
8. The process according to claim 7, wherein the phenoxypropylamine-type squalene synthase inhibitor is [3-(3-allyl-biphenyl-4-yloxy)-propyl]-isopropyl-amine, or a biologically

acceptable salt thereof, N-isopropyl-3-(4-acetamido-2-allylphenoxy) propylamine or N-methyl-N-isopropyl-3-(4-acetamide-2-allylphenoxy) propylamine.

9. The process according to claim 1 or 2, wherein the concentration of the said inhibitor is within the range that gives less than 50 % reduction of the cell growth under carotenoids

5 producing conditions.

10. The process according to claim 9, wherein the concentration of the said inhibitor is within the range that gives less than 30 % reduction of the cell growth under carotenoids producing conditions.

11. The process according to claim 1 or 2, wherein the cultivation is carried out at a pH in
10 the range from 4 to 8 and at a temperature in the range from 15 to 26 °C, for 24 to 500 hours.

12. The process according to claim 11, wherein the cultivation is carried out at a pH in the range from 5 to 7 and at a temperature in the range from 18 to 22 °C, for 48 to 350 hours.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/03742

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P23/00 C12N9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 09393 A (REYNOLDS TECHNOLOGIES INC ; BIOSOURCE TECH INC (US); HANLEY KATHLEE) 28 March 1996 (1996-03-28) page 1, line 16 - line 21 page 2, line 20 - line 25 page 7, line 3 - line 31 ----	1-12
X	DATABASE WPI Section Ch, Week 200346 Derwent Publications Ltd., London, GB; Class D16, AN 2003-485780 XP002250540 & JP 2002 300896 A (TOYOTA JIDOSHA KK), 15 October 2002 (2002-10-15) the whole document ----	1-12 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search

Date of mailing of the international search report

7 August 2003

22/08/2003

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/03742

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BROWN G R ET AL: "Phenoxypropylamines: a new series of squalene synthase inhibitors." JOURNAL OF MEDICINAL CHEMISTRY. UNITED STATES 13 OCT 1995, vol. 38, no. 21, 13 October 1995 (1995-10-13), pages 4157-4160, XP002250539 ISSN: 0022-2623 cited in the application the whole document</p> <p>---</p>	
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A	<p>US 5 182 208 A (JOHNSON ERIC A ET AL) 26 January 1993 (1993-01-26) cited in the application column 7, line 5 - line 8</p> <p>---</p>	

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Information on patent family members

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